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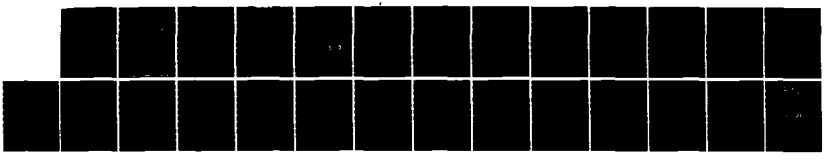

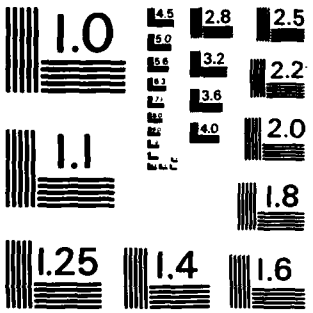

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Non-toxic synthetic adjuvants are under prime consideration for use in increasing the immune response of human beings. Three of microbial origin are the muramyl di-peptides (N-acetyl-muramyl-L-alanyl-D-isoglutamine and analogs, termed MDP), polyadenylic acid-polyuridylic acid complexes termed poly A·poly U) and the recently isolated monophosphoryl lipid A (termed MPL). While each of these has been demonstrated in animals to be active in increasing the immune response when given with the antigen, each also has been found to suppress this response when given one to several days before antigen. The enhancing actions of MDP and poly A·poly U have been well characterized. However, characterization of the suppressive phenomenon has been minimal, but is important to gain a responsible understanding of how these adjuvants regulate the immune response non-specifically. Accordingly, the experiments proposed during the tenure of this contract were undertaken to further our knowledge of how each of these adjuvants activate the suppressive arm of the immune response. The data are submitted in the form of four preprints and one reprint and

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permit the following main conclusions:

POLY A · POLY U

1. Poly A·poly inhibited antibody forming cells non-specifically when given 1-4 days before antigen, whereas poly I·poly C inhibited when given 1-6 days before antigen.

2. This suppression could be expressed in in vitro experiments by addition of surprisingly either T cell rich, B cell rich or adherent cell populations to their syngeneic normal cell counterparts, suggesting an unidentified cell may be contaminating each.

3. To determine whether an NK cell was contaminating the above 3 populations and was responsible for suppression, NK activity was removed with anti-asialo GM1 antibody without affecting the magnitude of the suppression.

4. Suppressive activity for both humoral and cell mediated immunity (MLR) was found and characterized in the serum of mice injected with poly A·poly U after 90 minutes.

5. Poly A·poly U increased non-specific resistance to Streptococcus pneumoniae and Pseudomonas aeruginosa when given 1-2 days before challenge with these microorganisms, despite the presence of antibody suppressing activity in the spleen at this time.

MURAMYL DI-PEPTIDES

1. A single injection of MDP either ip or iv, 1-2 days before antigen inhibited antibody forming cells by approximately 50%. This suppression lasted from 4-14 days with much individual variation.

2. Using derivatives of MDP it was shown that the muramyl grouping was not necessary for suppressive activity. The addition of an n-butyl ester grouping to the terminal carboxyl of the glutamine moiety of MDP did not increase the capacity to induce suppression.

3. Suppression could be transferred to syngeneic recipient mice with both adherent and non-adherent spleen cells. T cells were found to be the effector cell in the latter population.

4. Unlike poly A·poly U, MDP did not induce suppressive activity in the serum 90 minutes after injection.

5. Interleukin I activity was depressed 24 and 48 hr after MDP injection, while Il-2 activity became depressed later at 72 hr.

6. It was hypothesized that MDP initiates suppression in the macrophage population in the form of decreased Il-1 production, which in turn depressed Il-2 levels. The net result was a decrease in numbers of antibody forming cells.

MONOPHOSPHORYL LIPID A

1. A non-toxic monophosphoryl lipid A (MPL, Ribi) isolated from endotoxins of Gram negative bacteria was shown to exert an adjuvant action on both the helper and suppressor branches of the immune response. Thus, toxicity is not a requirement for the adjuvant action of bacterial endotoxins.

2. MPL restored antibody production in aging mice and in the endotoxin low responding mouse strains C3H/HeJ and C57Bl10/ScN. In addition, MPL induced suppression in the C3H/HeJ strain.

GENERAL

1. Poly A·poly U, MDP and LPS increased phagocytosis in macrophages from young mice, but appeared to suppress this activity in aging virgin mice.

2. Aging breeder mice on the other hand were activated to increased phagocytosis similar to young mice, suggesting hormonal factors may control certain reactivities to adjuvants.

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Suppression of Antibody Forming Cells

by Muramyl Di-peptides

by

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SUMMARY

I. Muramyl di-peptides.

A single injection of MDP when given 1-2 days before antigen to Balb mice suppressed PFC formation to thymus dependent but not thymus independent antigens approximately 50%. The suppression lasted from 4-14 days with marked variation in this parameter being exhibited by individual mice. The suppressive state could be transferred to normal (non-x-irradiated), syngeneic mice with both non-adherent and adherent spleen or peritoneal exudate cells with the non-adherent appearing more active than the adherent cells. Fractionation of the non-adherent population into T and B cells revealed the T cells to be suppressive while the B cells transfered an enhanced state. Removal of suppressive T cells reveals an enhancing action in the B cell population. Consequently, both enhancement and suppressive forces appear to be stimulated non-specifically by MDP with suppression dominating the total splenic activity when MDP is given before antigen.

Lower levels of interleukin 1 (Il-1) production by adherent cells from MDP injected mice as compared to PBS injected control mice were found after 24-48 hr. Interleukin-2 activity was decreased also, but this deficiency was exhibited only after 72 hr. These data suggest that MDP may result in suppressed macrophage function after 24 hr resulting in a lessened Il-2 production. Consequently, antibody production is decreased.

Multiple analogs of MDP have been synthesized in an attempt to relate their structure to the diverse functions exhibited by these compounds. Two of these which are currently undergoing intensive scrutiny for possible use in human beings were tested in our model for capacity to suppress antibody synthesis. They were N-acetyl-muramyl-L-alanyl-D-glutamine-n-butyl ester, termed murabutide and a desmuramyl deriviative, L-ala-D-isogln-L-ala-

$OCH_2CH(OH)CH_2O$-mycolate, termed triglymyc. Both exhibited suppressive activity when given 1-2 days before antigen. Suppression was not increased by addition of the butyl ester group to MDP. In addition, the muramyl group appeared not to be essential for induction of suppression since triglymyc was effective in this respect. The mechanism of suppressive action of these MDP adjuvants remains to be determined.

MATERIALS AND METHODS

Mice: 6-8 week old BALB/c mice of both sexes were used.

MDP: N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) was a gift from Dr. L. Chedid of the Institut Pasteur (Paris, France).

Antigens: Sheep erythrocytes (SRBC) were used as the T-dependent (TD) antigens, and trinitrophenyl (TNP)-Brucella abortus (Ba) as the T-independent (TI) antigen. The latter was a gift from Dr Uchiyama of Tokyo Women's Medical College (Japan) and was prepared according to the method of Mond et al. (6).

PFC assay: Four days after antigen injection in vivo the haemolytic plaque-forming cell (PFC) assay was performed by the monolayer plaque assay of Kennedy and Axelrad (1). The number of PFCs in 2×10^5 nucleated splenocytes per plates was counted.

Four days after in vitro tissue culture anti-SRBC PFC and anti-TNP Br. abortus PFC were assayed by the agar plaque technique according to the method of Uchiyama (2) and the number of PFCs per culture calculated.

In vitro tissue culture: Nucleated spleen cells (generally 1×10^7 cells per culture) were cultured with antigen in duplicate in tissue culture dishes (35 x 10mm: Nunc) at 37°C in 5% CO_2 for 4 days using Click's medium. containing 10% fetal calf serum (FCS) and 5×10^{-5} M 2 mercaptoethanol (2ME). As a source of interleukin-1 (IL-1), the supernatant fluids from adherent (AD) cells (derived from 1.5×10^7 whole spleen cells/culture) and incubated with

repurified lipopolysaccharide (LPS), E. coli 055:B5, Difco) were used. As a source of interleukin-2 (IL-2), the supernatant fluids of whole or fractionated spleen cells (5×10^6 cells per culture) incubated with concanavalin A (Con A, Miles-Yeda) were used. RPMI 1640 medium, used as culture medium, contained 5% FCS and 5×10^{-5} M 2 ME.

IL-1 and IL-2 assay: For detection of IL-1 activity, thymocytes from CBA/N mice which proliferate well in the presence of IL-1 were used, for Il-2 CTLL-2 cells, an IL-2 dependent T cell line, were used. Thymocytes of CBA/N mice (5×10^5 or 7.5×10^5 cells per culture) or CTLL-2 cells (1×10^4 cells per culture) were cultured in RPMI 1640 in triplicate in 96-well microculture plates (Nunc) in the presence of a 50% or 30% concentration of culture supernatants. The cultures were pulsed with 1 µCi of ^{3}H-thymidine 16 hours before the end of a 72 hour culture (thymocytes) or a 48 hour culture (CTLL-2 cells), and harvested and counted.

Separation of Adherent AD cells from nonadherent (NA) cells: AD cells were isolated from spleen cells by utilizing EDTA-reversible adherence of monocytes to surfaces of tissue culture flasks coated with the microexudate of baby hamster kidney (BHK) cells according to the method of Ackerman and Douglas (4) or by adhering monocytes to intact surfaces of tissue culture dishes and decanting the NA cells. These procedures were also used for depletion of macrophages. In some experiments residual macrophages in NA cells were removed by passage through Sephadex G-10 columns (8).

Separation of B cells and T cells: Whole or macrophage depleted spleen cells were incubated in plastic dishes coated with purified anti-immunoglobulin, and the adhering cells (B cells) harvested after nonadhering cells (T cells) were collected according to the method of Mage et al. (5).

Removal of T cells: T cells were removed by treating spleen cells with a monoclonal anti-theta antibody which was produced from hybridoma H013 cells (7) by Dr. Uchiyama, Tokyo, Japan. Whole spleen cells (2×10^7 cells per ml) in Hanks solution were reacted with the antibody at a final dilution of 1:200 at 4°C for 30 minutes, centrifuged, and suspended in young rabbit serum as a source of C' at a final dilution of 1:10. After incubation at 37°C for 30 minutes, the treated cells were washed 3X in Hanks solution.

Removal of Lyt-2.2$^+$ cells: Lyt-2.2$^+$ cells were removed by treating macrophage-depleted spleen cells with the monoclonal anti-Lyt-2.2 antibody (Cedarlane, Canada). After depleting macrophages by BHK-microexudate coated flasks, spleen cells (1×10^4 cells/ml) in Hanks solution were reacted with the antibody at a final dilution of 1:20 at 4°C for 40 minutes, centrifuged, and resuspended in young rabbit serum as a source of C' at a final dilution of 1:10. After incubation at 37°C for 60 minutes, the treated cells were washed three times in Hanks solution.

RESULTS

Suppression of in vivo immune response to SRBC by MDP:

BALB/c male mice were injected once with 300 µg of MDP by either the iv or ip route. Control mice received HBSS. One or two days later mice were injected ip with 1×10^8 SRBC, and the PFC responses assessed four days after antigen challenge (Table 1). Administration of a single injection of MDP by either route prior to antigen injection suppressed PFC responses significantly ($p < 0.01$).

Duration of suppression induced by MDP in vivo:

To determine the duration of the suppression induced by MDP in vivo, mice were injected with MDP or PBS one, two, five, ten and fourteen days prior to SRBC injection and PFC were responses examined on day 4 after antigen.

Injection of MDP on Day -1 or Day -2 induced marked suppression (87% and 51% respectively, $p < 0.05$, Table 2). Slight suppression was observed when MDP was injected 5, and 10 days before antigen (28 and 43% respectively). Restoration occurred gradually with much individual variation. Thus, in some mice a strong suppressive state was maintained for at least 10-14, days while others recovered within several days.

In vivo transfer of MDP-induced suppression by spleen cells:

One or two days after a single injection of 300 µg of MDP, spleen cells were prepared from MDP injected or noninjected mice and fractionated into adherent and nonadherent populations (experiment 1) and further separated into B cells and T cells (experiment II) (Table 3). When 1×10^7 of these cells were transferred into syngeneic normal mice along with antigen challenge, the PFC response in recipients was significantly suppressed by both adherent and nonadherent cell populations removed from MDP injected mice. Transfer of nonadherent cells appeared to be more effective than adherent cells. When the former were subdivided, T cells were found to be suppressive, while on the other hand B cells exerted an enhancing effect (Table 3). Whole spleen was not significantly suppressive, which probably reflected this balance between T suppressor cells and B cells.

In vivo transfer of in vitro MDP-induced suppression by peritoneal macrophages:

Since the adherent cells in spleens of MDP injected mice appeared to be marginally, but significantly capable of suppressing the normal PFC response, peritoneal macrophages treated with MDP in vitro were examined in this respect, also. Three days after stimulation by thioglycollate, peritoneal exudate cells were collected and 4×10^6 cells incubated for two hours with 3 µg/ml MDP in vitro. After washing, the cells were harvested and transferred

into syngeneic recipients. One or two days after macrophage transfer mice were challenged with SRBC (Table 4). The PFC responses were suppressed significantly by transfer of macrophages treated with MDP in vitro similar to that observed previously in vivo. Although, these results suggest macrophages may contribute to the total MDP-induced suppression, the presence of low numbers of T cells contaminating the adherent cells could not be ruled out.

Lack of Suppression of a Thymus-Independent Antigen.

To clarify the role of non-T cells in MDP induced suppression, spleen cells removed one or two days after the injection of 300 µg of MDP were cultured in vitro with either SRBC, a T-dependent antigen, or TNP-Ba a T-independent antigen (Table 5). The PFC response against SRBC was suppressed strongly by spleen cells from MDP injected mice ($p < 0.01$). However, the thymus independent antigen TNP-B. abortus was not affected.

Suppression induced by admixtures of cell preparations from MDP-, PBS- or normal-spleen cells:

To clarify which cell population was responsible for MDP-induced suppression, the PFC responses in vitro in mixed cultures of adherent cells and nonadherent cells prepared from MDP- or PBS-injected mice were examined (Table 6). In experiment (I) the combination of MDP-AD cells with either PBS-NA or MDP-NA cells was lower than when PBS-AD cells were mixed with PBS-NA or MDP-NA cells. (PBS-NA added to MDP-AD or to PBS-AD, 823 vs 1200 [$p < 0.05$], MDP-NA added to MDP-AD or to PBS-AD, 1247 vs 1560.) In contrast the magnitude of the response of MDP-NA cells added to AD cells, whether of PBS or MDP cells, was higher than that of PBS-NA cells. (MDP-NA or PBS-NA added to PBS-AD, 1560 vs 1300. MDP-NA or PBS-NA added to MDP-NA, 1247 vs 827 ($p < 0.05$). In experiment (II) similar results occurred in that PFC of normal NA

cells with MDP-AD cells was lower than that with PBS-AD cells, and PFC of normal AD cells with MDP-NA cells was higher than that with PBS-NA cells.

Interluekin-1 (IL-1) and interleukin-2 (IL-2) production by spleen cells from MDP-injected mice:

Production of interleukin-1 (IL-1) and interleukin-2 (IL-2) activity by spleen cells from MDP-infected mice also was examined and compared with those of normal spleen cells (Table 7). IL-1 production by MDP-macrophages (AD cells) was reduced significantly in 24h supernatant fluids in Exp. (II) and 48 h supernatant fluids of Exp. (I) and Exp. (II) when compared to PBS-macrophages.

IL-2 activity was assayed on an IL-2 dependent cell line CTLL (Table 8). There was no apparent difference between PBS- and MDP-whole spleen cells. Moreover no differences in Il-2 activity in nonadherent cells nor in Lyt-2.2$^+$ depleted nonadherent cells were observed after 48 hours incubation. However, after 72 hours supernatant fluid from all cell-fractions of MDP-treated spleen cells exhibited lower levels of IL-2 production than PBS cells ($p < 0.05$).

The relation between IL-1 production or IL-2 production and the PFC response in vitro:

In order to determine whether the MDP-induced PFC response was associated with a reduced IL-1 or IL-2 production, the anti-SRBC-PFC response of normal spleen cells was examined in the presence of culture supernatants from MDP-spleen cells similar to those used as sources of IL-1 in Table 7 and IL-2 in Table 8. Normal whole spleen cells cultured with LPS stimulated supernatant fluids of PBS-AD cells as a source of IL-1 showed a high PFC response, in contrast to fluids from MDP-AD cells ($p < 0.05$). No difference was observed in normal whole spleen cells cultured with Con A stimulated whole cell supernatant fluids as a source of IL-2. However, T cell depleted normal

spleen cells cultured with Con A-stimulated supernatant fluids from MDP-whole cells and of NA cells showed reduced PFC responses compared to those with fluids from PBS-whole or NA cells ($p < 0.05$). It is noteworthy that fluids from MDP-NA cells depleted of Lyt-2.2^+ cells exhibited an enhancing effect on PFC responses of T-cell depleted normal spleen cells ($p < 0.05$), Table 9.

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TABLE 1

Suppression of SRBC-PFC Response by MDP In Vivo.

Expt. No.	Pretreatment[a] Day	Substance	Route	Anti-SRBC-PFC/[b] 2×10^5 Spleen Cells	% Suppression
I	-2	HBSS	iv	556 ± 28	
	-2	MDP	iv	154 ± 18	72[d]
	-1	HBSS	iv	430 ± 36	
	-1	MDP	iv	283 ± 15	34[c]
II	-1	HBSS	ip	242 ± 1	
	-1	MDP	ip	126 ± 4	61[d]

a) BALB/c male (Exp. I) or female (Exp. I) mice injected with 300 µg MDP or HBSS one or two days prior to 1×10^8 SRBC ip.

b) PFC determined four days after SRBC injection. Values are the arithmetic means ± SEM.

c) $p < 0.05$.

d) $p < 0.01$.

TABLE 2

The Duration of Suppression Induced by MDP In Vivo.

Injection	PFC/5 x 10^6 cells[a] (MEAN ± SEM)	% Suppression (p =)
Day -1 MDP	26 ± 24	87 (.001)
PBS	206 ± 37	
Day -2 MDP	109 ± 11	51 (.001)
PBS	223 ± 25	
Day -5 MDP	179 ± 48	28 (.07)
PBS	247 ± 54	
Day -10 MDP	155 ± 107	43 (.07)
PBS	271 ± 38	
Day -14 MDP	262 ± 140	15 (.5)
PBS	309 ± 64	

a)
Balb/c mice injected on day 0 with 10^8 SRBC ip. PFC assayed on day +4.

TABLE 3

In Vivo Transfer of Suppression by Spleen Cells from MDP Injected Mice.[a]

Transferred Spleen Cell Populations	MDP-Treatment of Cell Donors	Expt. I	(% Suppression)[c]	Expt. II	(% Suppression)
Unfractionated Spleen Cells	—	573 ± 14		265 ± 10	
	+	528 ± 8	(8)	239 ± 10	(10)
Adherent Spleen Cells	—	498 ± 10		176 ± 8	
	+	435 ± 26	(13)	137 ± 5	(22)[e]
Nonadherent Spleen Cells	—	602 ± 4		104 ± 8	
	+	408 ± 9	(32)[f]	85 ± 3	(19)
Adherent Cells and Nonadherent Cells	—	548 ± 4		ND[d]	
	+	420 ± 15	(23)[f]	ND	
B cells	—	ND		116 ± 5.7	
	+	ND		193 ± 9.3	(-67)[f]
T cells	—	ND		140 ± 5	
	+	ND		54 ± 3	(61)[f]
In Vivo Control	—	542 ± 16		144 ± 6	
	+	393 ± 11	(28)[f]	102 ± 14	(29)

TABLE 3 LEGEND

a) BALB/c male mice were injected with or without 300 µg MDP iv (Expt. I) or ip (Expt. II). One day (Expt. II) or two days (Expt. I) later whole -, adherent (AD) -, nonadherent (NA) -, reconstituted adherent and nonadherent -, B cell - and T cell - spleen cell preparations were obtained from MDP injected or noninjected mice (three mice/group). 1×10^7 cells of each population were injected iv into normal BALB/c male mice simultaneously with 1×10^8 SRBC ip.

b) PFC values represent arithmetic means ± SEM.

c) % suppression as compared with noninjected mice in each group.

d) ND = not done.

e) $p < 0.05$

f) $p < 0.01$

TABLE 4

In Vivo Transfer of Suppression by Peritoneal Macrophages Treated with MDP In Vitro.[a]

Expt. No.	Macrophage Transfer	MDP-Treatment in vitro	Anti-SRBC-PFC/ 2×10^5 Spleen Cells	% Suppression
I	Day -2	-	248 ± 9	
		+	177 ± 3	29[b]
	Day -1	-	281 ± 3	
		+	147 ± 5	48[b]
II	Day - 1	-	193 ± 10	
		+	122 - 12	39[b]

a) Peritoneal macrophage cells were collected from three mice three days after thioglycollate injection, enriched by adherence to plastic and 4×10^6/2 ml cells incubated 2 hr with or without 3 µg/ml MDP. After washing twice, macrophages were harvested and transferred ip (5.7×10^6 viable cells in Expt. I, and 1.4×10^7 viable cells in Expt. II) into syngeneic normal mice. One or two days later the recipients were injected with 1×10^8 SRBC ip.

b) $p < 0.01$.

TABLE 5

In Vitro PFC Responses to SRBC and TNP-B. abortus

from MDP Injected Mice.[a]

Pretreatment	PFC/culture			
	SRBC	% Suppression)	TNP-B. abortus	(% Suppression)
Day -1 PBS	2117 ± 104		2525 ± 318	
Day -1 MDP	105 ± 15	(95)[b]	2350 ± 248	(7)
Day -2 MDP	175 ± 43	(92)[b]	2600 ± 71	(-)

a) Spleen cells were obtained from BALB/c female mice one or two days after ip injection of 300 µg MDP or PBS. The cells (5×10^6/culture) from each group were cultured with SRBC (1×10^7) or TNP-B. abortus (3×10^{-3} dilution). Four days later cultures were assayed from SRBC-PFC or TNP-PFC.

PFC values are the arithmetic means ± S.D.

b) $p < 0.01$.

TABLE 6

In Vitro PFC Responses by Combining Cell Populations from Mice Injected with MDP or PBS[a]

Expt. No.	Cell Combinations	Anti-SRBC PFC/culture[b]
I	PBS-NA + PBS-AD	1300 ± 22
	PBS-NA + MDP-AD	823 ± 45
	MDP-NA + PBS-AD	1560 ± 179
	MDP-NA + MDP-AD	1247 ± 68
	PBS-whole	725 ± 115
	MDP-whole	640 ± 60
II	Normal-NA + PBS-AD	805 ± 98
	Normal-NA + MDP-AD	155 ± 49
	Normal-AD + PBS-NA	235 ± 71
	Normal-AD + MDP-NA	675 ± 119
	PBS-whole	1500 ± 78
	MDP-whole	995 ± 114

a) 1×10^7 whole spleen cells were cultured 1 h and non-adherent (NA) cells separated from adherent (AD) cells. PBS- or MDP-NA cells were added to culture plates containing MDP- or PBS-AD cells (Exp. I). Normal-NA cells were added to culture plates containing PBS- or MDP-AD cells. PBS- or MDP-NA cells were added to culture plates containing normal-AD cells (Exp. II). The mixtures of AD cells and NA cells were cultured with 5×10^6 SRBC. PFC assay was performed 4 days later.

b) Mean PFC ± S.D. of triplicate assays.

TABLE 7

Interleukin-1 Production by Splenic Macrophages from Mice Injected with MDP or PBS

Expt. No.	Source of Il-1[a]	LPS (μg/ml)	CPM (Mean ± S.D.)[b] 24 h-SUP	48 h-SUP
I	PBS-AD cells	0	134 ± 40	371 ± 5[a]
		0.1	6098 ± 1457	6675 ± 2034
		1	8066 ± 1480	11880 ± 1188
	MDP-AD cells	0	239 ± 49	369 ± 226
		0.1	3832 ± 1256	3852 ± 1506
		1	6212 ± 1119	6917 ± 1111[c]
II	PBS-AD cells	0	865 ± 265	253 ± 3
		0.1	1786 ± 149	784 ± 136
		1	2357 ± 482	2028 ± 731
	MDP-AD cells	0	924 ± 764	300 ± 299
		0.1	155 ± 95[c]	187 ± 117[c]
		1	478 ± 132[c]	495 ± 101[c]
	LPS alone without cells	0	107 ± 67	
		0.1	220 ± 39	
		1	228 ± 60	

a) Source of IL-1: 1.5×10^7 whole spleen cells of MDP or PBS injected BALB/c mice were incubated for 1 h and separated into adherent and non-adherent cells. Adherent cells were cultured with 0, 0.1, 1 μg/ml of LPS for 24 or 48 h and the supernatants collected as sources of IL-1 activity.

b) Assay of IL-1: 5×10^5 thymocytes of CBA/N mice were cultured in triplicate in microplates with supernatants added as 50% in Exp. (I) or 30% in Exp. (II). The cultures were plused with 1 μCi of ^{3}H-thymidine 16 h before the cultures were terminated at 72 h.
CPM values are mean CPM ± S.D. of triplicate assays.

c) Significant at $p < 0.05$.

TABLE 8

Interleukin-2 Production by Spleen Cells from Mice Injected with MDP or PBS.

Expt. No.	Source of IL-2[a]	Con A (μg/ml)	CPM (Mean ± S.D.)[b] 24 h	48 h	72 h
(I)	PBS-whole cells	0	101 ± 76	162 ± 47	ND
		3	118195 ± 13210	55924 ± 8472	
	MDP-whole cells	0	166 ± 83	380 ± 110	
		3	119893 ± 9019	48146 ± 4598	
	Con A alone without cells	3	147 ± 25		
(II)	PBS-whole cells	3	ND	67187 ± 2061	7162 ± 973
	MDP-whole cells	3		68718 ± 2352	4327 ± 532[c)]
	PBS-NA cells + Normal-ND cells	3		79497 ± 2659	48216 ± 1630
	MDP-NA cells + Normal-AD cells	3		80365 ± 2599	36165 ± 4928[c]
	PBS-(Lyt.-2.2$^-$) NA cells + Normal-AD cells	3		82880 ± 7062	60804 ± 4682
	MDP-(Lyt.-2.2$^-$) NA cells + Normal-AD cells	3		83867 ± 7671	46337 ± 3526[c]
	Con A alone without cells	3			132 ± 50

TABLE 8 LEGEND

a) Source of IL-2: 5×10^6 whole spleen cells of MDP- or PBS-injected BALB/c mice were incubated with 3 μg of Con A in culture plates for 24, 48 or 72 hours. In Exp. (II) 5×10^6 PBS or MDP-NA cells depleted of AD cells with BHK-microexudate-coated flasks (in one group) or depleted further of Lyt.-2.2 positive cells by treatment with anti-Lyt.-2.2-serum and complement (in another group) were added to normal-AD cell monolayers. The cell mixtures were cultured with 3 μg of Con A for 48 or 72 hours and the supernatants collected as sources of IL-2 activity.

b) Assay of IL-2: 1×10^4 CTLL cells were cultured in triplicate in microplates with supernatants added to 30%. The cultures were pulsed with 1 Ci of ^{3}H-thymidine 16 hours before termination of culture at 48 hours.

CPM values are mean CPM ± S.D. assayed in triplicate.

c) Significant at $p < 0.05$.

TABLE 9

In Vitro PFC Response of Normal Spleen Cells Cultured with Supernatants PBS or MDP Treated Spleen Cells

Expt. No.	Supernatant from	Spleen cells cultured	SRBC/PFC/culture[c]	% Suppression[d]
(I)[a]	LPS Stimulation			
	PBS-AD cells	Normal whole spleen cells	3457 ± 114	
	MDP-AD cells		2610 ± 78[e)]	25
	LPS without cells		(2680 ± 96)	
	Con A Stimulation			
	PBS-Whole cells	Normal whole spleen cells	1617 ± 143	
	MDP-Whole cells		1610 ± 134	
	Con A without cells		(1817 ± 126)	
(II)[b]	Con A Stimulation			
	PBS-Whole cells	T-cell depleted normal spleen cells	1867 ± 69	
	MDP-Whole cells		1377 ± 71[e]	26
	PBS-NA cells (+ Normal AD cells)		3610 ± 158	
	MDP-NA cells (+ Normal AD cells)		2640 ± 118[e]	27
	PBS (Lyt.-2.2$^-$) NA cells (+ Normal AD cells)		2997 ± 101	
	MDP (Lyt.-2.2$^-$ NA cells (+ Normal AD cells)		4267 ± 131[e]	(-42)

TABLE 9 LEGEND

a) In Expt. (I) the supernatants (SUPs) were the same as the 24 h SUP of Expt. (II) in Table 7 and Exp. (I) in Table 8. 200 µl of SUPs of AD cells stimulated with LPS or 20 µl of SUPs of whole cells stimulated with Con A were added to 9.2×10^6 normal whole spleen cells and cultured with 5×10^6 SRBC for 4 days.

b) In Expt. (II) the SUPs were the same as the 72 h SUP of Expt. (II) in Table 8. 1.0×10^7 T cell-depleted normal spleen cells treated with anti-θ serum were cultured with 50 µl of SUPs and 5×10^6 SRBC for 4 days.

c) Mean PFC ± S.D.

d) % suppression as compared with control cultured with PBS-SUP in each group.

e) Significant at $p < 0.05$.

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